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Use of Copper as a Trigger for the *in Vivo* Activity of *E. coli* Laccase CueO: A Simple Tool for Biosynthetic Purposes

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Laccases are multi-copper oxidases that catalyze the oxidation of various electron-rich substrates with concomitant reduction of molecular oxygen to water. The multi-copper oxidase/laccase CueO of *Escherichia coli* is responsible for the oxidation of Cu^{+} to the less harmful Cu^{2+} in the periplasm. CueO has a relatively broad substrate spectrum as laccase, and its activity is enhanced by copper excess. The aim of this study was to trigger CueO activity *in vivo* for the use in biocatalysis. The addition of 5 mM $CuSO_4$ was proven effective in triggering CueO activity at need with minor toxic effects on *E. coli* cells. Cu-treated *E. coli* cells were able to convert several phenolic compounds to the corresponding dimers. Finally, the endogenous CueO activity was applied to a four-step cascade, in which coniferyl alcohol was converted to the valuable plant lignan (−)-matairesinol.

Introduction

Laccases belong to the superfamily of multi-copper oxidases, which are found in insects, fungi, bacteria, and plants.[1] They catalyze the one-electron oxidation of electron-rich phenolic and nonphenolic compounds with concomitant four-electron reduction of molecular oxygen to water. Generally, laccases possess a wide substrate spectrum, do not require any external cofactors, and produce water as the only by-product. These characteristics make them interesting tools for biotechnological applications ranging from wastewater bioremediation, the decolorization of industrial dyes and the degradation of pharmaceutical micropollutants to manufacturing biologically active compounds.[2] Consequently, diverse laccases from many organisms have been discovered, characterized and widely applied as biocatalysts.[3]

Bacterial laccases have recently attracted attention, as they offer several advantages in comparison to fungal or plant laccases.[4] Generally higher thermal and pH stability of bacterial laccases, as well as lower sensitivity towards inhibitors in comparison to eukaryotic ones have been reported. A number of bacterial laccases have been identified, isolated and characterized.[5] However, the expression of recombinant laccases in *Escherichia coli* might be affected by incomplete incorporation of Cu ions into the apoprotein and by interference with activity of the endogenous multi-copper oxidase CueO.[6]

For the past 20 years, the structure and function of CueO, whose former gene name was *yacK*, have been intensively investigated.[7] As part of the homeostasis system in *E. coli*, CueO is responsible for the oxidation of Cu^{+} to the less harmful Cu^{2+} in the periplasm, thereby preventing Cu^{+} from entering the cytoplasm.[8] Typically for multi-copper oxidases, CueO possesses a Type 1 copper site and a Type 2/Type 3 trinuclear copper cluster that together are responsible for substrate oxidation and oxygen reduction.[9] The binding site of CueO is hidden by a methionine-rich helical region that was suggested to prevent the access of bulky organic substrates, determining enzyme specificity as a cuprous oxidase.[10] At low environmental copper concentrations, CueO is expressed and folded into the cytoplasm and then transported to the periplasm via the twin arginine pathway as apoprotein.[8] Once in the periplasm, copper ions assemble as metal cofactors to apo-CueO determining structural stabilization and catalytic activation of the enzyme. In this regard, the addition of external Cu^{2+} ions, like copper sulfate ($CuSO_4$), has been demonstrated to readily activate the enzyme, leading to the full incorporation of four copper ions in the T1, T2 and T3 centers.[8,11] Previous studies have revealed that CueO has a rather broad substrate spectrum with low activity *in vitro*, yet the activity increased in the presence of Cu^{2+} excess.[7a] Recently, CueO was overexpressed in *E. coli* and its activity was tested *in vitro* for oxidative coupling of phenolic compounds.[12]

Within this study, the use of the *in vivo* oxidase activity of CueO triggered by the addition of external copper ions to *E. coli* cells for oxidative phenolic coupling was investigated. This approach represents a suitable solution for biocatalytic applications. For instance, in cascade reactions, laccase activity could be switched on only when needed and without host manipulation. Oxidative coupling of various phenolic compounds with *E. coli* cells was tested under copper induced stress. A major focus was set on the dimerization of coniferyl alcohol to the lignan (±)-pinoresinol, because in a previous study from our group, dimers of coniferyl alcohol were formed by exploiting an *E. coli*-based whole-cell biocatalyst, expressing the recombinant CgL1 laccase from *Corynebacterium glutamicum*.[13] As a proof of

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concept, we developed an *in vivo* four-step cascade to prove the applicability of this approach. Endogenous CueO activity was switched on by $CuSO_4$ addition, and combined with heterologously expressed pinoresinol-lariciresinol reductase from *Forsythia intermedia* (FiPLR) and secoisolariciresinol dehydrogenase from *Podophyllum pleianthum* (PpSDH). Utilizing this setup, up to 5 mM coniferyl alcohol were efficiently converted to the valuable lignan (−)-matairesinol.

Results and Discussion

Copper toxicity and its influence on *E. coli* BL21(DE3) cells

Copper is a fundamental element for cell life and enzymatic functions, yet its toxicity to biological systems has been widely recognized.[14] Specifically, lethal cell damaging related to environmental excess of copper has been associated with the displacement of iron from iron–sulfur proteins leading to the inactivation of enzymes involved in TCA cycle and pentose phosphate pathway, but also with the formation of oxygen reactive species (ROS).[11a,15] Although the occurrence of oxidative damage on DNA was excluded, the addition of $CuSO_4$ to *E. coli* directly correlated with superoxide generation in the cells. Yet, $CuSO_4$ is often supplemented within heterologous expression of bacterial laccases in *E. coli* to achieve complete copper loading in the target enzymes.[16] For these reasons, we first tested the effect of increasing concentrations of $CuSO_4$ on growing *E. coli* cells and their viability. Concerning aerobic growth conditions in complex media, sensibility of *E. coli* cells towards copper has been reported to occur at a millimolar concentration; specifically, in LB medium the reported minimum inhibitory concentration (MIC) was 3.5 mM $CuSO_4$.[16b,17]

Cell cultivation was carried out in TB medium in the presence of increasing $CuSO_4$ concentrations (3, 5, 10, 15, 20 or 30 mM), which revealed mild growth inhibition after addition of 3, 5, 10 and 15 mM compared to the negative control without copper added (Figure 1A). Within 48 h cell growth, the OD_{600} value of the negative control reached ~12, while under addition of up to 15 mM $CuSO_4$ OD_{600} values of ~10 were recorded. As the major difference between LB and TB media is the addition of glycerol as carbon source and phosphate buffer to temper pH variations, these results suggest that the growth of well-fed and metabolically active *E. coli* cells is indeed influenced by copper ion concentrations >3 mM, yet copper tolerance appears higher than the previously reported MIC in LB medium.

Cell viability was additionally investigated by counting the number of colony forming units (CFUs) on agar plates. Aliquots taken immediately after addition of 3, 5, or 10 mM $CuSO_4$ ($t=$ 0 h) indicate that copper significantly affects the number of CFU as cell viability apparently drops by ~30–40% as compared to the negative control (Figure 1B). However, after 24 h incubation time, the cell viability is recovered, since similar numbers of CFU were counted for the samples supplemented with up to 10 mM $CuSO_4$ (Figure 1B). To explain this behavior, metabolic shock with following adaption of the cellular environment is hypothesized. For instance, iron–sulfur dehydratase clusters, and among these the fumarase A involved in the TCA cycle, have been reported to be inhibited by Cu ions. Nonetheless, the activity of this enzyme was shown to be protected from Cu ions if the active site was already occupied by the substrate malate, but also restored once the inhibiting copper was removed.[15] Because of this, it is possible that *E. coli* cells metabolism is harmed by $CuSO_4$ addition at first, and then recovers once copper ions are cleansed or detoxified by the homeostasis systems.

15 mM $CuSO_4$ appears as a threshold value for *E. coli* resilience: Similar OD_{600} values to those recorded with 3–10 mM $CuSO_4$ were achieved (Figure 1A), but the viability on agar plates dropped by ~90% after 24 h of incubation (Figure 1B). Differently, cell growth was strongly inhibited when 20 or 30 mM $CuSO_4$ concentrations were used (Figure 1A), and no colonies were detected on agar plates after 24 h.

For further analysis, *E. coli* cells were analyzed by microscopy. Cells from the negative control and experiments with 3 and 5 mM $CuSO_4$ appeared evenly distributed over the slide surface with no clear differences in cells morphology. Concerning 10 mM CuSO4, *E. coli* cells were evenly distributed on the

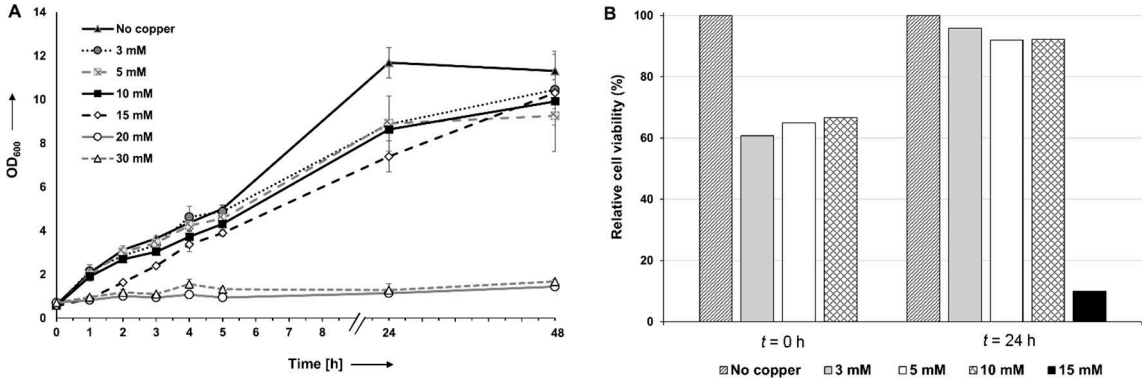

Figure 1. Effect of $CuSO_4$ on *E. coli* BL21(DE3) cell growth and viability. A) Cell growth in complex medium (terrific broth). B) *E. coli* cell viability on 3, 5, 10 and 15 mM $CuSO_4$ immediately after copper addition (time point 0 h), and after 24 h. Cell viability 0 h after addition of 15, 20, and 30 mM and 24 h after the addition of 20, and 30 mM $CuSO_4$ are not shown because no colonies were detected on agar plates. The number of CFUs from the negative control without $CuSO_4$ was set as 100%. Shown data result from two independent measurements.

glass slide, but the cells appeared smaller (Figure S1 in the Supporting Information).

Dimerization of phenolic compounds by $CuSO_4$-triggered CueO

The oxidase activity of CueO depends on the presence of environmental Cu ions and it has been proven to be active on diverse laccase substrates *in vitro*.[12,18] In light of this, we chose 5 mM $CuSO_4$ to trigger CueO activity in *E. coli* cells for the oxidation of phenolic compounds with subsequent dimerization. Generally, dimers of phenolic compounds have been reported to gain enhanced biological activities compared to the monomers.[19] Several compounds including 2,6-dimethoxyphenol (2,6-DMP), ferulic acid, coniferyl alcohol, tyrosol, *trans*-resveratrol and 17β-estradiol were chosen for tests for the following reasons. 2,6-DMP and ferulic acid have been described as substrates for CueO that catalyzes their oxidation *in vitro*.[12] Other compounds are reported substrates for different laccases. One-electron oxidation of phenolic compounds catalyzed by a laccase leads to the formation of phenoxy radicals which couple to dimers or/and oligomers. Dimers of 2,6-DMP were reported to possess higher antioxidant capacity than the substrate itself.[20] The reaction with 2,6-DMP (*m/z* 173 $[M+H+H_2O]^+$) oxidation turned bright yellow and three products could be detected by LC/MS. The first product with *m/z* 307 $[M+H]^+$ was identified as the desired dimer, whereas the two others were characterized by *m/z* 293 $[M+H–CH_3]^+$ and *m/z* 278 $[M+H–2CH_3]^+$, thus suggesting the loss of one or two methyl groups, respectively (Figure S2 and Table S1).[21]

Concerning ferulic acid (*m/z* 196 $[M+2H]^+$), whose dimers have been reported to increase its antioxidant activity, four products were detected. Amongst these, the analysis of the ionization patterns suggested two of them being the β–β and β–5 dimers (*m/z* of 387 $[M+H]^+$; Figure S3), coherently to those described in the literature. The MS data of the other detected products suggest the loss of CHO_2 by the dimers (*m/z* 341; Figure S3 and Table S2).[22]

Starting with coniferyl alcohol, the formation of four coupling products was observed. These compounds were identified as (±)-erythro/threo-guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers, (±)-dehydrodiconiferyl alcohol, and (±)-pinoresinol (Figure S4 and Table S3), based on the knowledge achieved from a previous work from our group.[23] Together with the observed coupling products, coniferyl aldehyde (*m/z* 179 $[M+H]^+$) was identified as resulting from the activity of endogenous dehydrogenases in *E. coli*.[13,24] Among the coupling products, the β–5 dimer dehydrodiconiferyl alcohol and the β–β dimer (±)-pinoresinol have been reported to possess interesting features: dehydrodiconiferyl alcohol was proven to exhibit anti-adipogenic, anti-oxidant and anti-inflammatory activities, and also appeared active in osteoblasts differentiation process;[25] (±)-pinoresinol is known for anti-inflammatory, anticancer and anti-microbial activities.[26]

Three products were formed during conversion of resveratrol (*m/z* 229 $[M+H]^+$). However, only one out of three showed *m/z* 455 $[M+H]^+$ corresponding to resveratrol dehydrodimers reported in the literature.[27]

Because of poor ionization, tyrosol could be identified only in the UV/Vis spectrum; here, one conversion product was observed (Figure S5). No activity towards 17β-estradiol could be detected under the tested conditions, which might be due to high reduction potential or large size of this substance which make it inappropriate as a substrate for CueO.

Optimization of endogenous CueO activity in *E. coli* for coniferyl alcohol coupling

Influence of oxygen supply and copper concentration: With the idea of further development of a *E. coli* whole cell biocatalyst featuring the multi-copper oxidase activity of CueO, we chose coniferyl alcohol as starting substrate to produce (±)-pinoresinol, which is the main precursor for higher-lignans biosynthesis.[28] As multi-copper oxidases rely on oxygen for their activity, in the first test, the conversion of coniferyl alcohol was carried out in tubes with open or sealed lids.

Coniferyl alcohol was efficiently converted (~85%) in tubes with open lid when 5 mM $CuSO_4$ were added to the cells. Contrary, in closed tubes low conversion (~4%) was observed even after $CuSO_4$ addition (Figure 2A). No substrate conversion was observed in tubes with open lids without added copper (Figure 2B). These results can be explained based on the mode of action of the two major components of copper homeostasis in *E. coli*, namely the *cue* system and the *cus* system.[11a,17] According to the literature, the *cue* system is identified as the primary homeostasis system, either in aerobic or anaerobic conditions, and its genes *copA* and *cueO* are described as basally expressed with mild-overexpression of ~12-fold enhancement in case of increasing copper stress. The efflux *cus* system is only strongly expressed by ~800-fold, if the primary *cue* system is overwhelmed. Nonetheless, in anaerobic conditions, the *cus* system is described to take over the primary role, enhancing copper efflux supported by CopA, whereas CueO is not involved in copper tolerance anymore.[11b] All in all, as multi-copper oxidase require oxygen for catalysis, this might represent a complementary explanation for the limited CueO activity which was observed under limited air availability.

Next, we tested lower and higher $CuSO_4$ concentrations to verify its influence on laccase activity of *E. coli* cells. CueO specific activity *in vitro* was reported at 3–4 mM $CuSO_4$ concentration;[8] for this reason we chose diverse copper concentrations ranging from 1–10 mM. Our results revealed that increasing copper concentrations resulted in increased coniferyl alcohol conversion with 5 mM $CuSO_4$ leading to ~70% and 10 mM $CuSO_4$ to 100% conversion (Figure 3A). Aiming to reduce copper usage and in order to be able to compare the reaction conditions, we used 5 mM $CuSO_4$ in further experiments because this concentration resulted in satisfactory substrate conversion.

In a previous work from our group, coniferyl alcohol dimerization to (±)-pinoresinol via *E. coli* biotransformation was achieved using heterologously expressed bacterial laccases

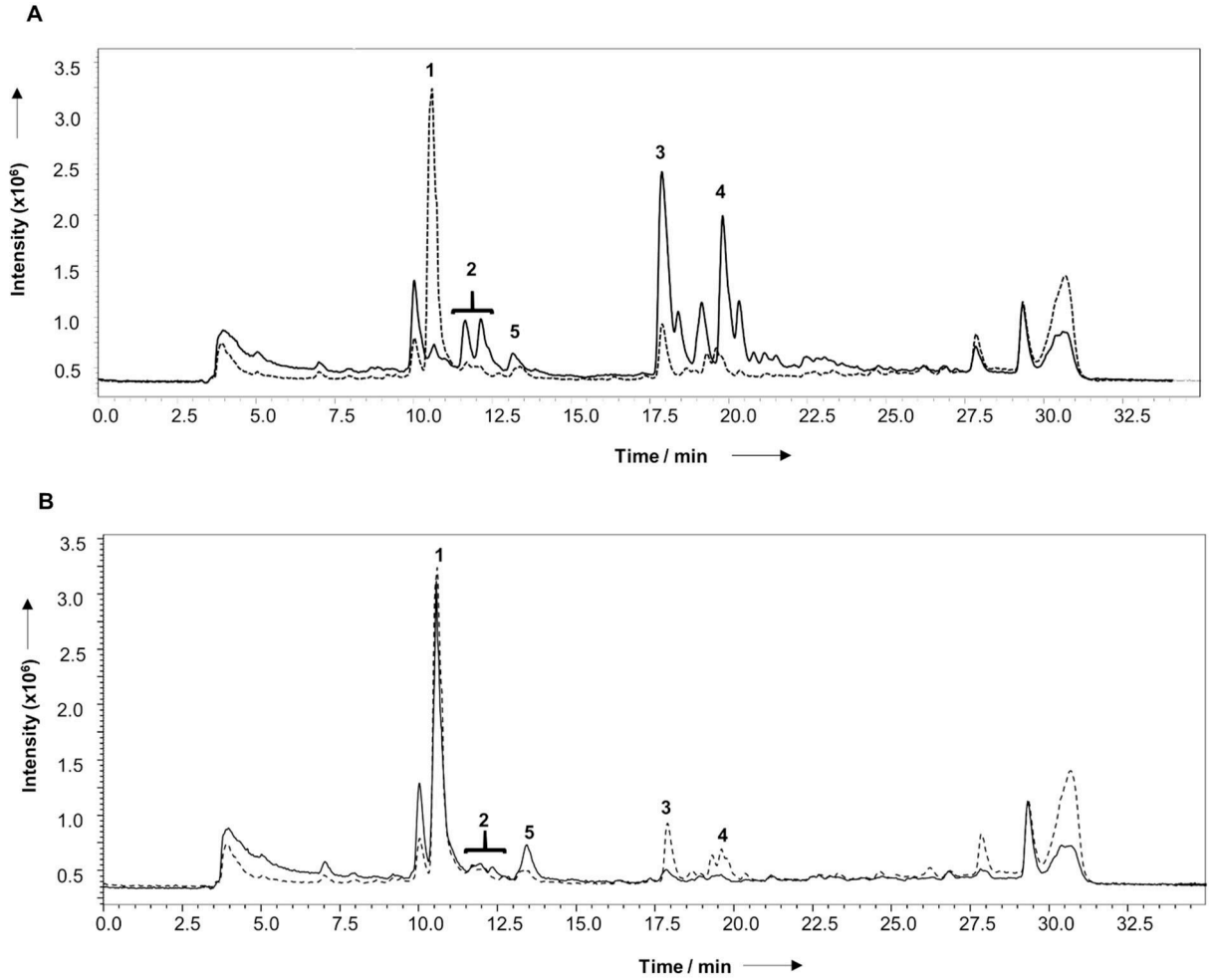

Figure 2. Comparison of coniferyl alcohol conversion catalyzed by *E. coli* cells under copper-induced stress in open and closed tubes. A) The reaction performed with 5 mM $CuSO_4$ under aeration (—) is plotted against the sample with 5 mM $CuSO_4$ in limited air availability (---). B) The sample with 5 mM $CuSO_4$ in limited air availability (---). is plotted against the control sample in an opened tube with no copper added (—). 1: coniferyl alcohol, 2: (±)-erythro/threo-guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers, 3: (±)-dehydrodiconiferyl alcohol, 4: (±)-pinoresinol, 5: coniferyl aldehyde.

including CgL1 from *C. glutamicum*.[23] For this reason, we decided to compare the endogenous CueO-system with heterologously expressed CgL1 and quantify the achieved (±)-pinoresinol concentration. Complete conversion of 200 μM coniferyl alcohol was achieved after 2 h with CueO whereas ~93% conversion was observed with CgL1, resulting in 65 and 40 μM (±)-pinoresinol, respectively. However, after 4 and 24 h the amount of the desired product (±)-pinoresinol decreased in both cases. This is no surprise, because phenolic compounds like (±)-pinoresinol are accepted by laccases as substrates as well, leading to the formation of oligo- or polymers.[29]

Complete substrate depletion by CueO was not expected, since the addition of 5 mM $CuSO_4$ in the previous experiments did not lead to full conversion. A reason for better performance might be related to cell permeability. Whereas in the previous experiments *E. coli* resting cells were used directly after cultivation, in these experiments cells were frozen after harvesting and thawed prior usage, allowing higher membrane permeability and increased substrate accessibility in the cell,[30] which likely resulted in improved conversion.

CueO led to higher (±)-pinoresinol concentration after 2 h compared to CgL1 (Figure 3B), which is probably due to the different reduction potentials of these enzymes. More precisely, the reduction potential reported for CueO is 440 mV, but 260 mV for CgL1, resulting in faster monomer oxidation by CueO, but also faster oxidation of the product (±)-pinoresinol (Figure 3B).[23,31]

In order to prove if this approach could be applied to higher substrate concentrations, a tenfold higher coniferyl alcohol load (2 mM) was tested, and 5 mM $CuSO_4$ was applied to trigger CueO activity. In this case, complete substrate conversion was achieved after 16 h, resulting in a (±)-pinoresinol concentration of 243 ± 15 μM. This value has to be put in context with the product distribution, as the mechanism of bimolecular phenoxy radical coupling typical for multi-copper oxidases prevents regio- and stereoselectivity, leading always to a mixture of products.[32] In nature, stereoselective phenoxy radical coupling

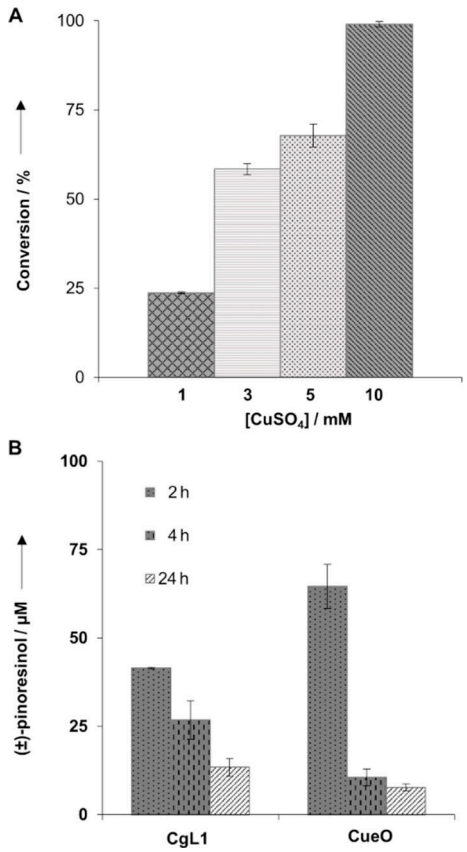

Figure 3. A) Influence of copper sulfate [mM] on CueO activity with 200 µM substrate. B) Comparison of (±)-pinoresinol concentrations measured at different time points with heterologous Cgl1 and endogenous CueO starting with 200 µM substrate.

is directed by the presence of so-called dirigent proteins, whose physiological role is to prevent random coupling of radicals occurring after the one-electron oxidation of monolignols like coniferyl alcohol performed by laccases or other oxidizing enzymes.[32] In absence of dirigent proteins, coniferyl alcohol radicals have been described to generate spontaneously (±)-dehydrodiconiferyl alcohol, (±)-erythro/threo-guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers and (±)-pinoresinol at a ~10:3:5 ratio.[33] Coherently, in our experiments, coniferyl alcohol radicals were most frequently coupled to (±)-dehydrodiconiferyl alcohol (~65%), while (±)-erythro/threo-guaiacylglycerol 8-*O*-4′-coniferyl alcohol ethers and the desired product (±)-pinoresinol set to ~8% and ~27%, respectively (Figure S6). This means that when starting with 2 mM coniferyl alcohol resulting in 1 mM of coupling products that are available after oxidative dimerization, ~250 µM (±)-pinoresinol is the theoretically possible titer. Thus, the detected (±)-pinoresinol concentration appears close to the theoretically achievable optimum.[33,34]

Validation of the roles of CueO and copper ions in coniferyl alcohol coupling: So far, it was assumed that CueO is responsible for coniferyl alcohol coupling, being readily activated by the addition of copper ions. As copper is widely used as catalyst to perform several reactions,[35] aerobic copper-mediated generation of coniferyl alcohol radicals was tested applying 3, 5 or 10 mM $CuSO_4$ with 2 mM substrate in phosphate buffer without cells or isolated enzymes. Approximately 25% depletion was observed with 3 mM $CuSO_4$ and ~35% with both 5 and 10 mM. However, only minor amounts of (±)-dehydrodiconiferyl alcohol appeared as a clearly distinguishable product (Figure S7A). In the light of these results, $CuSO_4$ could be addressed to partake in the reaction independently from CueO. However, this activity doesn't seem crucial *in vivo*, since 5 and 10 mM $CuSO_4$ applied to *E. coli* cells led to >99% substrate conversion (Figures 3A, 4 A, and S7B). Moreover, as already described before, *E. coli* possesses several homeostatis systems capable of interacting with free copper ions, which – together with specific and unspecific chelators – will immediately diminish the amount of available copper that could trigger radical formation and consequent coupling.[11a,36] In order to further validate this hypothesis, the *E. coli* BL21(DE3) strain was compared to an *E. coli* BL21(DE3) strain with deleted *cueO* gene (Δ*cueO*). While 99% conversion of 2 mM coniferyl alcohol was reached using *E. coli* BL21(DE3) resting cells after $CuSO_4$ addition, the *E. coli* BL21 (DE3) Δ*cueO* strain reached only 17% conversion, endorsing the role of CueO (Figure 4A). Low conversion observed with the Δ*cueO* strain is not surprising: apart from the above described non-enzymatic reaction it is also likely that other enzymes may take over the activity of CueO within the cell rather than free copper.[36] For instance, endogenous *E. coli* peroxidases, namely Prx01 and Prx02, have been described capable to catalyze the oxidative coupling of coniferyl alcohol to (±)-pinoresinol.[37] As copper cytotoxicity correlates with oxidative stress, the presence of H_2O_2 necessary for peroxidase activity seems plausible.

We also compared common copper sources like $CuSO_4$ and $CuCl_2$ to exclude other ions to influence the desired activity and confirm the role of copper ions as leading actor. As a matter of fact, variable responses of laccase activities have been associated to different ions. Specifically, sulfate salts have been reported in some cases to enhance laccase activity, whereas Cl^- ions often show an inhibiting effect.[38] The addition of 5 mM

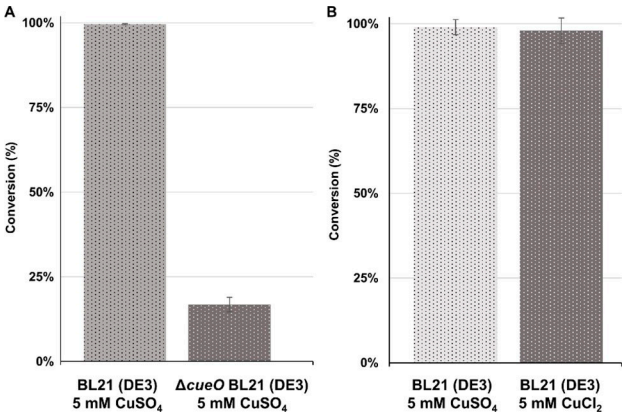

Figure 4. Conversion of coniferyl alcohol. A) Comparison of conversions catalyzed by *E. coli* BL21(DE3) and by *E. coli* BL21(DE3) Δ*cueO*; 5 mM $CuSO_4$ were added to growing cells, and conversion was performed with resting cells. B) Effect of different copper salts on coniferyl alcohol conversion by *E. coli* BL21(DE3); 5 mM $CuSO_4$ or $CuCl_2$ were added to resting cells together with 2 mM coniferyl alcohol.

$CuSO_4$ or $CuCl_2$ resulted in >99% conversion of 2 mM coniferyl alcohol in both cases, thus suggesting no influence of sulfate or chloride on CueO activity (Figure 4B).

Timing of copper sulfate addition: Aiming to prove copper addition to switch on CueO activity at need, $CuSO_4$ supplementation was tested at different time points of strain cultivation. Generally, $CuSO_4$ was added to growing cells at ~0.6 OD_{600}, whereas substrate conversion was performed with resting cells after cell harvesting. As an alternative, we tested substrate conversion with growing cells by adding coniferyl alcohol and $CuSO_4$ together at ~0.6 OD_{600} during cell growth. The growing cell approach led only to very low substrate conversion, and product detection was possible by mass spectrometry in single ion monitoring only (Figure S8). Prolongation of cell growth up to 72 h and lowering the incubation temperature from 30 to 25 °C did not result in any improvement, which demonstrates that resting cells are superior for the desired purpose.

In a third approach, cells were grown, harvested, and copper was added to resting cells together with the substrate. Especially with this setup, we aimed to prove that $CuSO_4$ acts indeed as the trigger inducing activity of CueO. In this case, the mechanism of copper ions loading in CueO is probably different compared to the growing cells. Nevertheless, utilizing this setup, efficient coniferyl alcohol depletion (~99%) was observed and (±)-pinoresinol concentrations were similar to those achieved when copper was added during cell growth (244 ± 53 μM).

A possible explanation for the diverse results achieved with growing and resting cells may be the interplay between oxygen availability and copper content leading to different cellular environments that influence catalytic activity or expression. Previous studies have shown that during growth phase oxygen consumption by *E. coli* cells is sensibly higher than with resting cells,[39] therefore CueO activity in growing cells might be limited because oxygen is consumed for other metabolic needs. Moreover, the heterologous overexpression of CueO in *E. coli* and its oxidizing activity *in vitro* were shown to correlate with cell growth under aerobic or micro-aerobic conditions.[12] According to that study, higher levels of CueO were achieved under fully aerobic conditions although CueO isolated from micro-aerobic cultures showed higher oxidizing activity.

Complementarily, the different outcome of conversions with growing or resting cells might be due to other physiological parameters like the cell concentrations applied for biotransformation.[40] To assess this, we performed whole-cell biotransformations of 2 mM coniferyl alcohol with resting cells normalized to cell wet weights of 70, 50, 30 and 20 g/L. These cell density values were chosen because after biotransformation with growing cells, the determined cell wet weights were usually ranging from 20–30 g/L.

After 4 h, 43 and 55% conversion was observed at cell concentrations of 70 and 50 g/L, respectively. With 30 and 20 g/L, substrate depletion achieved 36 and 17% (Figure S9). However, coniferyl aldehyde produced from coniferyl alcohol by endogenous *E. coli* dehydrogenase was detected as well, and its concentration was higher when lower cell concentrations were used. In particular, 14% coniferyl aldehyde was observed with 70 g/L, and ~20% with 50, 30 and 20 g/L, whereas 8–10% (±)-pinoresinol was detected for every tested condition. After 21 h, ~98% conversion of coniferyl alcohol was achieved regardless of the amount of cells (Figure S10). Despite the high amounts of coniferyl aldehyde spotted at 4 h, after 21 h coniferyl aldehyde is barely detected, and is probably reduced again to coniferyl alcohol, which is then dimerized. Given that different cell densities did not result in higher conversion values after 21 h, these results suggest that in resting cells a certain amount of "ready-to-go" CueO is present to efficiently convert coniferyl alcohol, whereas in the set up with growing cells, the continuous basal expression of CueO during cell growth may not be sufficient to convert the same concentration of coniferyl alcohol under the tested conditions, even with moderate overexpression as reported previously.[11b] However, metabolic engineering tools such as the usage of stronger promoters could provide useful tools for the improvement of the growing cell setup.[41] Furthermore, cell permeability could be another reason leading to the different behaviors of resting and growing cells. As already proven within the previous experiments, the integrity of the cell membrane might limit substrate diffusion and, as a consequence, the conversion.[30,42]

Application of CueO triggered activity to a cascade reaction

In order to demonstrate its usefulness, the CueO triggered activity was applied to a four-step cascade. The resting whole-cell approach was used to convert coniferyl alcohol to (−)-matairesinol. Within this cascade two additional enzymes are involved: Pinoresinol-lariciresinol reductase from *F. intermedia* (FiPLR) which catalyzes the two-step reduction of (+)-pinoresinol to (−)-secoisolariciresinol via the intermediate (+)-lariciresinol, and secoisolariciresinol dehydrogenase from *P. pleianthum* (PsSDH), which catalyzes the oxidation of (−)-secoisolariciresinol to (−)-matairesinol.

Three different setups were tested with 5 mM coniferyl alcohol. First, a "one-cell one-pot" setup was applied (Figure 5A): CueO activity was triggered by adding 5 mM $CuSO_4$ to *E. coli* C41(DE3) co-expressing FiPLR and PsSDH. After 20 h conversion, coniferyl alcohol was converted to ~89%, however, a high amount of coniferyl aldehyde was detected (~64% of the total products), while the desired compounds were poorly detectable (Figure S11).

Additionally, two mixed "two-cells one-pot" approaches were tested. *E. coli* BL21(DE3) cells with 5 mM $CuSO_4$ added during the growth phase was appointed as "*module one*", whereas *E. coli* C41(DE3) co-expressing FiPLR and PpSDH was set as "*module two*". In the first trial, the cascade was realized by mixing *modules one* and *two* at the same time immediately before substrate addition (Figure 5B). After 20 h reaction time, ~78% substrate depletion was achieved and again coniferyl aldehyde appeared as a distinct signal among the products accounting to 22%. Nevertheless, higher signals for the products of coniferyl alcohol coupling were detected, and the desired final product (−)-matairesinol (*m/z* 359 $[M+H]^+$, 341

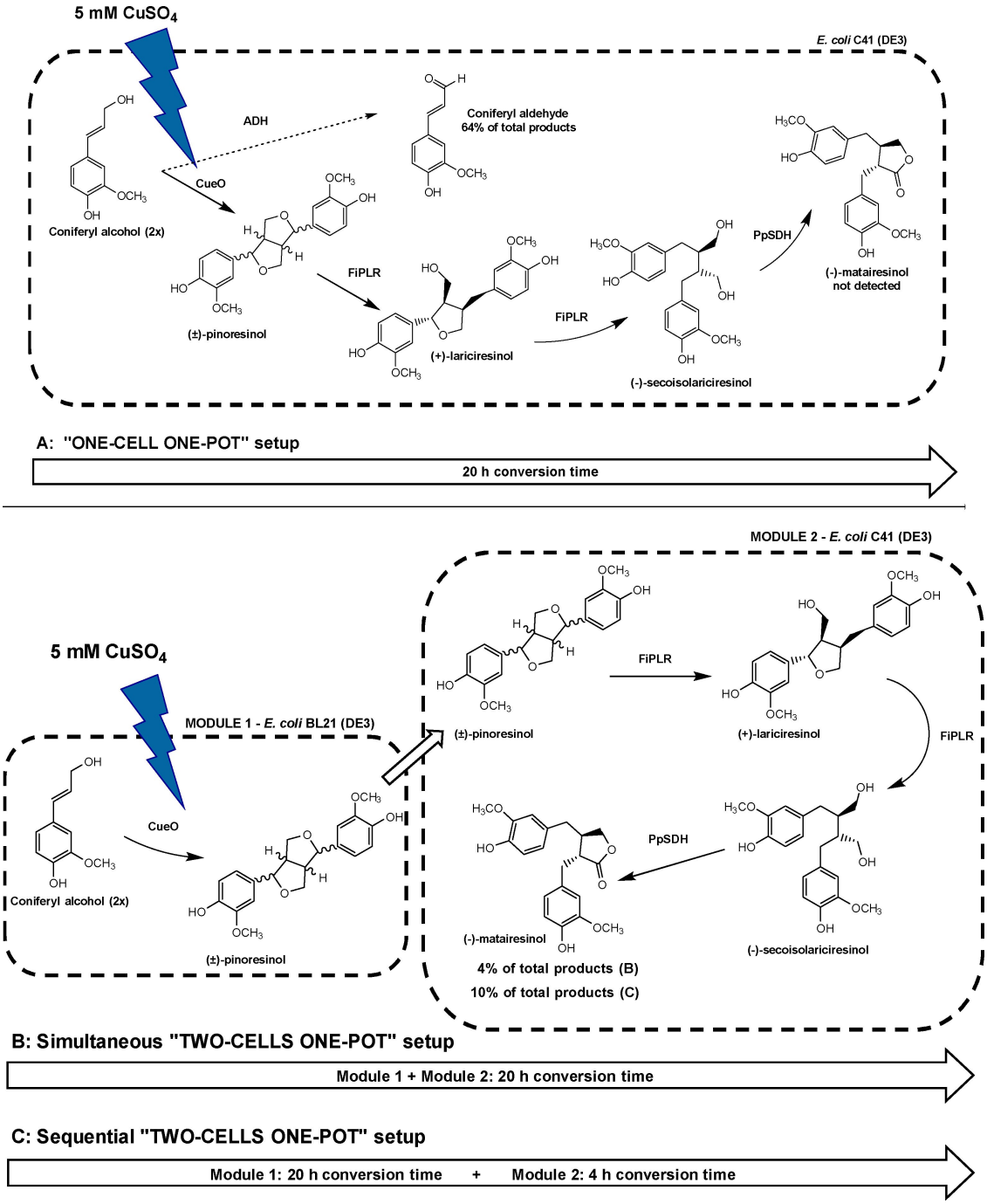

Figure 5. Overview of the three setups used for multistep transformation of coniferyl alcohol to (−)-matairesinol. A) "One-cell one pot" setup: *E. coli* C41(DE3) cells harboring FiPLR and PpSDH supplemented with 5 mM $CuSO_4$; side product coniferyl aldehyde: ~64%, no desired product (−)-matairesinol detected; B) Simultaneous "two-cells one-pot" setup: *E: coli* BL21(DE3) cells supplemented with 5 mM $CuSO_4$ were mixed with *E: coli* C41(DE3) cells harboring FiPLR and PpSDH prior to the start of the reaction; side product coniferyl aldehyde: 22%, desired product (−)-matairesinol: 4% of the total products. C) Sequential "two-cells one-pot" setup: *E: coli* BL21(DE3) cells supplemented with 5 mM $CuSO_4$ were incubated for 20 h alone to convert coniferyl alcohol to (±)-pinoresinol, afterwards *E. coli* C41(DE3) cells harboring FiPLR and PpSDH were added, and the incubation time was prolonged for 4 h. With this setup, production of coniferyl aldehyde was reduced to 2%, whereas (−)-matairesinol accounted for 10% of the total products, corresponding to the theoretically expected value for this cascade.

$[M+H-H_2O]^+$) accounted to 4% of the total products (Figure S12).

In the sequential "two-cells one-pot" approach, the conversion of 5 mM coniferyl alcohol with *module one* run for 20 h; afterwards *module two* was added and conversion was then prolonged for 4 h (Figure 5C). Overall, this approach appeared to be most promising, as coniferyl alcohol was efficiently converted within the first 20 h (97%) with only marginal

production of coniferyl aldehyde (2%) and the distribution of the coupling products according to those of the previous experiments, with (±)-pinoresinol representing 20% (Figure S12). After addition of *module two*, approximately 50% of (±)-pinoresinol were consumed, indicating that only one enantiomer was converted by FiPLR, as expected based on a previous report.[13] A few residues of the intermediate lariciresinol (*m/z* 383 $[M+Na]^+$) were detected (~2%), but no (−)-secoisolariciresinol fragments (*m/z* 363 $[M+H]^+$, 345 $[M+H-H_2O]^+$). The final product (−)-matairesinol corresponded to ~10%. As discussed above starting with 5 mM substrate concentration, the phenolic coupling occurring on coniferyl alcohol will result in 2.5 mM concentration of the products, approximately 500 μM of which is racemic (±)-pinoresinol. This means that the observed 10% (−)-matairesinol would correspond to ~250 μM. Indeed, the final product was quantified via internal standard calibration, resulting in 247 ± 11 μM (−)-matairesinol, which corresponds well to the theoretically expected value. Altogether, the sequential "two-cells one-pot" approach seems to be the most suitable setup for further investigation.

Overall, the balance between coniferyl alcohol and coniferyl aldehyde obviously influences *E. coli* cell performance in various setups. Indeed, the more aldehyde was observed the less coniferyl alcohol coupling products were detected. Endogenous *E. coli* dehydrogenases which might be responsible for these oxidation/reduction reactions use nicotinamide species as cofactors,[43] meaning that their function is influenced by the redox and energetic state of the cell. On the other hand, the ratio between coniferyl alcohol and coniferyl aldehyde was affected by the presence of heterologous FiPLR and PpSDH, which are known to use NADPH and NADH as cofactors, respectively.[44,45] It means that both enzymes exploit cell metabolism for their function in parallel with endogenous dehydrogenases, altering the energetic state and probably competing for the same cofactors. This might explain a high ratio of coniferyl aldehyde observed in the "one-cell one-pot" setup. When endogenous ADHs were separated from the heterologous FiPLR and PpSDH in the "two-cells one-pot" setups, the balance was shifted towards coniferyl alcohol, which was converted to pinoresinol in the *module one*. In the *module two* pinoresinol was converted in three steps to (−)-matairesinol (Figure 5B and C). The lowest coniferyl aldehyde concentration and the highest (−)-matairesinol concentration could be achieved when the two *modules* were separated not only spatially but also added at different time points. This observation supports the above suggestion that in the *E. coli* cells expressing FiPLR and PpSDH coniferyl alcohol is easier oxidized to coniferyl aldehyde and thus remains inaccessible for the cascade.

Conclusion

In conclusion, the *in vivo* activity of the *E. coli* endogenous multi-copper oxidase CueO was triggered by the addition of copper salts. Treated *E. coli* cells were proven effective to oxidize several phenolic compounds leading to the corresponding dimers. In these experiments it was possible to switch on CueO activity arbitrarily at need, with the further aim to include host endogenous activity into a whole-cell biocatalyst featuring a four-step cascade to produce (−)-matairesinol. Starting with 5 mM coniferyl alcohol 247 μM final product was formed, which corresponds to the theoretically expected value.

Notwithstanding its limitations, this approach offers a simplification in the landscape of the whole-cell biocatalysis for multi-enzyme cascades and is generally an interesting approach to develop powerful microbial cell factories.

Experimental Section

Bacterial strains, Cultivation, and Expression

E. coli BL21(DE3) cells were purchased from Novagen (Merck), whereas *E. coli* BL21(DE3) with *cueO* gene deletion (Δ*cueO*) were generated via TargetTron mutagenesis kit (Sigma–Aldrich) as described previously[6b] by introducing a kanamycin resistance gene. As *cueO* is an endogenous *E. coli* gene, cells were transformed with pET16a or pET24b vectors (Merck) with no foreign genes by heat shock procedure to ensure antibiotic resistance as selection marker preventing contamination. Heterologous expression of *Corynebacterium glutamicum* laccase CgL1, cloned in pET16b was performed as described previously.[13] More details are provided in Tables S4 and S5.

Pre-cultures were prepared in 5 mL LB medium supplemented with the appropriate antibiotic and inoculated with one colony from LB-agar plates or from a preserved cryo-stock, always in biological duplicate at least. Cells were then grown overnight (O/N) at 37 °C, 180 rpm. From these pre-cultures, 500 μL were inoculated in 50 mL fresh Terrific broth medium (TB) supplemented with an appropriate antibiotic and cells grew at 37 °C, 180 rpm until an OD_{600} value of ~0.6. The heterologous expression of foreign genes was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cultures. Cells were then incubated at 30 °C for 21–22 h, 140 rpm (Multitron, Infors HT, Switzerland). For the proof-of-concept biotransformation of coniferyl alcohol to (−)-matairesinol, *E. coli* C41 (DE3) OverExpress (Lucigen) cells were co-transformed with the vector pCDFDuet-1, harboring pinoresinol-lariciresinol reductase from *F. intermedia* (GenBank AAC49608; FiPLR) and secoisolariciresinol dehydrogenase from *P. pleianthum*/*Dysosma pleiantha* (GenBank AHB18702; PpSDH). Protein expression was induced with 0.5 mM IPTG and carried out at 25 °C for 48 h, 120 rpm (Multitron, Infors HT, Switzerland).

Harvest, Normalization of Cell Density and Whole-Cell Biotransformation Setup

After cell growth and gene expression, cultures were harvested via centrifugation for 30 min, 4 °C, 3220 *g* and the resulting pellets either directly used or stored at −20 °C. In the latter case, prior to further experiments, cells were thawed and their density normalized to 70 g/L (unless stated otherwise) using 50 mM phosphate buffer, KPi (80% K_2HPO_4, 20% 50 mM KH_2PO_4, pH 7.5) supplemented with 500 mM D-glucose and 0.1 mM IPTG. Whole-cell biotransformation were performed in 500 μL reaction volume composed the normalized cell suspension and substrate solution in 2% (*v/v*) DMSO. For screening purposes, 200 μM substrate concentration was used and samples incubated in 1.5 mL Eppendorf tubes with open lids at 25 °C, 1500 rpm for 24 h unless stated otherwise.

Substrates tested with *E. coli* with induced CueO are listed in Table S6. For further investigation and product quantification, substrate concentration was increased to 2 mM and conversion after 2, 4, 16 and 24 h were analyzed. Conversion of coniferyl alcohol by CueO was investigated using both growing and resting cells. Concerning conversions with growing cells, the setup was the same as the expression experiments; once the cultures reached an OD_{600} of ~0.6, 5 mM $CuSO_4$ and 2 mM coniferyl alcohol–2% (*v*/*v*) DMSO- were added. Incubation was performed at 25 or 30 °C, for 21 to 72 h at 140 rpm (Multitron, Infors HT, Switzerland).

Three setups were tested for the proof-of-concept biotransformation of coniferyl alcohol to (−)-matairesinol using the resting cells approach. In every case, cells were resuspended in 50 mM phosphate buffer KPi (80% K_2HPO_4, 20% 50 mM KH_2PO_4, pH 7.5) supplemented with 500 mM D-glucose and 0.1 mM IPTG with cell wet weight normalized to 70 g/L. A "one-cell one pot" was prepared consisting of 10 mL of *E. coli* C41(DE3) cell suspension co-expressing FiPLR and PpSDH and 5 mM coniferyl alcohol added together with 5 mM $CuSO_4$ to trigger CueO activity. Conversions were carried out in 100 mL Erlenmeyer flasks at 25 °C, 200 rpm for 20 h.

Alternatively, a mixed "two-cells one pot" was applied. Concerning the simultaneous setup, 5 mL of *E. coli* BL21(DE3) supplemented with 5 mM $CuSO_4$ during growth phase were mixed with 5 mL *E. coli* C41(DE3) co-expressing FiPLR and PpSDH. 5 mM coniferyl alcohol was added and the conversions was carried out in 100 mL Erlenmeyer flasks at 25 °C, 200 rpm for 20 h.

In a sequential setup, the first step of the reaction was performed with 10 mL *E. coli* BL21(DE3) supplemented with 5 mM $CuSO_4$ during growth phase (*module one*), with 5 mM coniferyl in 100 mL Erlenmeyer flasks at 25 °C, 200 rpm for 20 h. Subsequently, 10 mL of *E. coli* C41(DE3) with co-expressed FiPLR and PpSDH were added as *module two* and the reaction run for 4 h.

Copper Addition

The addition of $CuSO_4$ as a trigger for enzymatic activity was performed during cell growth phase, or in case of resting cells after harvest but prior substrate addition. $CuSO_4$ concentrations of 1, 3, 5, and 10 mM were tested. The same $CuSO_4$ concentrations were used to test copper mediated coniferyl alcohol oxidation and subsequent radical coupling. Reactions were performed in 50 mM phosphate buffer KPi (80% K_2HPO_4, 20% 50 mM KH_2PO_4, pH 7.5), 500 µL reaction volume with 2 mM substrate in 2% DMSO, *v*/*v*). Experiments were performed in technical triplicate. Samples were incubated at 25 °C, 1500 rpm for 24 h.

Metabolite Extraction and Analysis

Prior to the extraction, 200 µM of internal standard ferulic acid or sesamin were added to the samples appointed for quantitative analysis. Metabolites were extracted twice using 1 mL of ethyl acetate, the resulting organic phase was then evaporated under reduced pressure. After evaporation, samples were resuspended in methanol (MeOH, 99.9% LC/MS grade, Fischer Scientific) for LC/MS analysis.

Both qualitative and quantitative analysis were performed by liquid chromatography coupled with mass spectrometry (LC/MS) measurements on LCMS-2020 system (Shimadzu, Tokyo, Japan) equipped with a Chromolith® Performance RP-18e column (100× 4.6 mm, Merck). More details are provided in Tables S7 and S8). Samples appointed for quantitative analysis were made in technical and biological duplicate at least.

Copper Toxicity and Cell Viability Tests

Increasing concentrations of $CuSO_4$ were added to investigate copper toxicity on cells growth and viability. 400 mL of fresh TB medium were inoculated with 4 mL of overnight culture of *E. coli* BL21(DE3) and cells grew to an OD_{600} of 0.6 at 37 °C, 180 rpm (Multitron, Infors HT, Switzerland). At the OD_{600} of 0.6, cells culture was divided in 100 mL aliquots, $CuSO_4$ at concentrations of 3, 5, 10, 15, 20, and 30 mM was added, and cells were further incubated at 37 °C, 180 rpm. Growth was followed over 24 h by measuring OD_{600}. Viability of *E. coli* cells after copper addition was investigated by taking 40 µL of cell suspension straight after copper addition (t = 0 h) and/or after 24 h incubation time (t = 24 h). Aliquots were diluted to get the same cell amount, corresponding to an OD_{600} value of 0.5, and spread on LB agar plates supplemented with 30 µg/mL kanamycin.

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Conflicts of Interests

The authors declare no conflicts of interests.

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